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Pharmacogenetic Discovery in CALGB (Alliance) 90401 and Mechanistic Validation of a *VAC14* Polymorphism That Increases Risk of Docetaxel-Induced Neuropathy

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Abstract

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Previous Reports: This study was presented in part at the 2013 American Society of Clinical Oncology Annual Meeting. This report contains original material that has not been previously reported.

Purpose—Discovery of single nucleotide polymorphisms (SNPs) that predict a patient's risk of docetaxel-induced neuropathy would enable treatment individualization to maximize efficacy and avoid unnecessary toxicity. The objectives of this analysis were to discover SNPs associated with docetaxel-induced neuropathy and mechanistically validate these associations in preclinical models of drug-induced neuropathy.

Experimental Design—A genome-wide association study was conducted in metastatic castrate-resistant prostate cancer patients treated with docetaxel, prednisone and randomized to bevacizumab or placebo on CALGB 90401. SNPs were genotyped on the Illumina HumanHap610-Quad platform followed by rigorous quality control. The inference was conducted on the cumulative dose at occurrence of grade 3+ sensory neuropathy using a cause-specific hazard model that accounted for early treatment discontinuation. Genes with SNPs significantly associated with neuropathy were knocked down in cellular and mouse models of drug-induced neuropathy.

Results—498,081 SNPs were analyzed in 623 Caucasian patients, 50 (8%) of whom experienced grade 3+ neuropathy. The 1000 SNPs most associated with neuropathy clustered in relevant pathways including neuropathic pain and axonal guidance. A SNP in *VAC14* (rs875858) surpassed genome-wide significance ($p=2.12 \times 10^{-8}$ adjusted $p=5.88 \times 10^{-7}$). siRNA knockdown of *VAC14* in stem cell derived peripheral neuronal cells increased docetaxel sensitivity as measured by decreased neurite processes ($p=0.0015$) and branches ($p<0.0001$). Prior to docetaxel treatment *VAC14* heterozygous mice had greater nociceptive sensitivity than wild-type litter mate controls ($p=0.001$).

Conclusions—*VAC14* should be prioritized for further validation of its potential role as a predictor of docetaxel-induced neuropathy and biomarker for treatment individualization.

Keywords

docetaxel; paclitaxel; pharmacogenetics; genome-wide association study; heritability; single nucleotide polymorphism; chemotherapy-induced peripheral neuropathy; *VAC14*; peripheral neuronal cell model; animal model

Introduction

The taxane class includes three US Food and Drug Administration (FDA)-approved chemotherapeutic agents, paclitaxel, docetaxel and cabazitaxel, that have activity in a variety of solid tumors including lung, breast, ovarian, gastric, and prostate cancers. Taxanes bind to and stabilize microtubules, ultimately inhibiting the mitotic phase of cell cycle development(1). The taxanes and other microtubule-targeting chemotherapeutic agents (e.g., vinca alkaloids) commonly induce peripheral neuropathy of varying severity(2). Chemotherapy-induced peripheral neuropathy (CIPN) often presents as a combination of paresthesia and dysesthesia, and can progress to irreversible loss of balance and dexterity with continued treatment(3). Due to the progressive nature of the toxicity and its potential effect on long-term quality of life(4), CIPN can necessitate treatment discontinuation, even in the context of active tumor control.

Patient demographic (i.e., race and history of neuropathy) and treatment (i.e., cumulative dose and drug exposure) factors have been associated with CIPN(5-8), but there are currently no effective methods for predicting CIPN risk and/or severity. Genome-wide association studies (GWAS) of paclitaxel and vincristine-induced CIPN confirm that genetic variation modifies patients' risk(9-12). The discovery and validation of single nucleotide polymorphisms (SNPs) that influence risk of docetaxel-induced peripheral neuropathy would be of clinical benefit, enabling identification of high-risk patients in whom taxane use should be avoided if possible. SNPs that modulate risk of docetaxel-induced neuropathy have been reported from candidate gene studies(13-15); however, no study has taken a genome-wide approach. Based on the previous success of GWAS in discovering SNPs that affect paclitaxel-induced neuropathy, a similar analysis was conducted in a cohort of docetaxel-treated patients to identify genetic predictors of docetaxel-induced neuropathy.

GWAS was performed in a large, prospectively enrolled, chemotherapy-naïve cohort of metastatic castrate-resistant prostate cancer (mCRPC) patients who were treated with up to two years of docetaxel and prednisone, with half randomized to concurrent bevacizumab(16). As these metastatic patients are at high risk of early treatment discontinuation due to disease progression and/or death, which precludes occurrence of neuropathy, a competing-risks adjusted statistical model was utilized(17, 18). Following discovery of a SNP that surpassed genome-wide significance, pharmacogenetic replication between the results of a previously conducted GWAS of paclitaxel-induced neuropathy(9) and this GWAS of docetaxel-induced neuropathy and mechanistic validation of that gene in cellular and animal models were attempted to determine whether genetic predictors of CIPN were similar between the taxanes and to potentially validate clinically useful genetic predictors of docetaxel-induced sensory peripheral neuropathy

Methods

Patients and toxicity

Cancer and Leukemia Group B (CALGB/Alliance) 90401 was a double-blinded phase III trial that equally randomized men with hormone-refractory prostate cancer to receive docetaxel and prednisone with or without bevacizumab for up to two years(16). All patients enrolled in the CALGB 90401 parent study who provided IRB-approved informed consent for a pharmacogenomic substudy (CALGB 60404) were eligible for this GWAS. Briefly, patient eligibility included histologically documented adenocarcinoma of the prostate that had progressed while the patient was on hormone deprivation therapy. Relevant exclusion criteria included prior chemotherapy or anti-angiogenesis therapy or clinically significant (grade 2+) peripheral neuropathy. Toxicity data were collected by the Alliance Statistics and Data Center at each treatment cycle on standardized forms that mandated reporting of grade 3+ peripheral sensory neuropathy, as defined by National Cancer Institute Common Toxicity Criteria for Adverse Events (NCI CTCAE) version 3.0. Additionally, one case was reported as a grade 3 cranial neuropathy event described as “sensory-facial.” Only neuropathy that was considered by the clinician to be possibly, probably, or definitely attributable to treatment, and occurred within 30 days of docetaxel administration, was included in the analysis.

Docetaxel treatment

All patients received docetaxel 75 mg/m² infused over 1 hour on day 1 of each 21-day cycle with 8 mg oral dexamethasone 12, 3, and 1 hour prior to infusion. Patients on both arms also received 5 mg oral prednisone twice daily and were randomized to 15 mg/kg bevacizumab or placebo by intravenous infusion on day 1 of each cycle. Use of growth factor, aspirin, anti-emetics and luteinizing-hormone releasing hormone agonists was under the discretion of the treating physician. Docetaxel administration was held for neutropenia (absolute neutrophil count <1,500 cells/mm³) and the dose was decreased in increments of 10 mg/m² for hepatic dysfunction, neurotoxicity, gastrointestinal toxicity or febrile neutropenia. The protocol mandated discontinuation of docetaxel treatment if the patient required more than two docetaxel dose decreases or in the event of specific toxicities or confirmed cancer progression.

Genotyping

A 10-mL sample of whole blood was collected from all patients enrolling on the pharmacogenomic substudy prior to initiation of protocol treatment. Genotyping and genetic quality control were similar to that previously reported in the CALGB 40101 GWAS(9). Genotyping was performed on the HumanHap610-Quad Genotyping BeadChip (Illumina Inc., CA, USA) at the RIKEN Center for Genomic Medicine (Yokohama City, Japan). Genotype data is available at dbGap (http://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs001002.v1.p1) SNPs that were known indeterminate (n=4,106) or unreliable loci (Tech Note: Infinium® Genotyping Data Analysis, 2007) and patients with SNP call rate <95% were excluded. Eigensoft version 3.0 was used to visualize genetic ancestry for the 790 evaluable patients to identify a genetic European population (n=623) in which the analysis was conducted. Additional SNPs were excluded for low call rate (<99%), low minor allele frequency (<0.05), p-value of Hardy-Weinberg distribution <10⁻⁸ or non-autosomal loci, leaving 498,081 SNPs for analysis (Supplementary Figure 1).

Cellular Sensitivity Experiments

Commercially available human induced pluripotent stem cell (iPSC)-derived peripheral neurons (Peri.4U) were purchased from Axiogenesis (Cologne, Germany) and plated at 1 × 10⁴ cells/well in 100 µL media as per manufactures' instructions. Figure 3A illustrates the scheme for this experiment. Dharmacon Accell technology (GE Dharmacon, Lafayette, CO, USA) was applied at 4 hours post-plating using 1 µM human si *VAC14* SMARTpool or the non-targeting control (NTC). 24 hours later, transfection media was exchanged for media containing 0.17% DMSO vehicle control and either docetaxel (LKT Laboratories Inc., St. Paul, MN) or paclitaxel (Sigma-Aldrich, St. Louis, MO). The drugs were prepared in a darkened hood by dissolving powder in 100% DMSO to obtain a stock solution of 58.4 mM. Stock drug was serially diluted in media for final dosing concentrations ranging from 10 pM to 1 µM, increasing by factors of ten. Control wells were treated with 0.17% final concentration of DMSO to match drug treatments. After 24 or 48 hour drug treatments, neurons were stained with 0.3 ng/mL Hoechst 33342 (Sigma-Aldrich) and 1.5 µg/mL Calcein AM (Molecular Probes, Life Technologies Inc.) and imaged at 10× magnification using an ImageXpress Micro imaging microscope (Molecular Devices, LLC, Sunnyvale,

CA) at the University of Chicago Cellular Screening Center. Neurite changes were determined by individual cell measurements of relative total neurite outgrowth, relative number of processes, relative number of branches, mean/median/max process length, relative cell body area, relative straightness and relative mean outgrowth intensity. The data represents triplicate experiments making measurements on more than 1000 cells per treatment as previously described(19). Two wells of transfected cells were collected per timepoint using the Cells-to-Ct kit (Ambion, Life Technologies Inc., Carlsbad, CA) and reversed transcribed as per kit instructions. Quantitative real time reverse transcription polymerase chain reaction (qRT-PCR) was performed using Taqman primers (Fisher Scientific LLC, Hanover Park, IL) for *VAC14* (Hs00947931_m1) and compared to human beta-2-microglobulin (NM_004048.2) housekeeping gene at 24, 48 and 72 hours post-transfection (as previously described; (19).

Mouse Sensitivity Experiments

Mice bred for *VAC14* heterozygosity (*VAC14*^{+/-}) and *VAC14* wild-type litter mates (*VAC14*^{+/+}) on C57/BL6 background were obtained from Lois Weisman at University of Michigan and acclimated to living conditions at University of Maryland(20). Mice 3-5 months of age were randomly assigned to docetaxel or placebo treatment, with 6-7 mice in each treatment arm. The entire experiment was conducted separately in male and female mice. Intraperitoneal administration of docetaxel 2 mg/kg or saline vehicle was administered on days 1, 3, 5, and 7.

Behavioral testing for the development of increased mechanical sensitivity took place at baseline (prior to treatment), day 5 (after two doses), day 8 (24 hours after the fourth dose), and weekly for 3 additional assessments. Mechanical sensitivity was measured in mice from both treatment arms simultaneously by a blinded individual using von Frey filaments, as previously described(21). Briefly, mice were placed in individual Plexiglas® cubes on an elevated wire mesh platform and allowed to acclimate for approximately one hour, when grooming and exploration behaviors ended. A series of von Frey filaments (Touch Test Sensory Evaluator Kit, myNeurolab.com, St. Louis, MO), with bending forces of 0.04-2.0 g, were applied to the plantar surface of the left hind paw until the filament just bent and was held in place for 5 seconds or until the mouse withdrew its paw. A positive response was defined as a brisk withdrawal, with or without shaking or licking, of the hind paw during or immediately upon removal of the filament application. Each filament was tested 5 times on each hind paw starting with the 0.4 g filament. If the 0.4 g filament elicited 3 positive responses out of 5 trials, then testing continued moving downward through the series to the 0.04 g filament and the number of withdrawals was recorded for each filament. If the 0.4 g filament did not elicit 3 positive responses, then testing continued moving upward through the series to the 2.0 g filament and the number of withdrawals was recorded for each filament. Threshold was defined as the filament with the lowest bending force that elicited at least 3 positive responses out of 5 trials.

Statistics

The primary endpoint for GWAS analysis was the cumulative docetaxel dose (mg/m²) at first report of treatment-related grade 3+ sensory peripheral neuropathy. Patients who did not

complete two years of therapy due to progression/death, treatment-terminating adverse event, or any other reason were informatively censored at the cumulative docetaxel dose received; therefore, the statistical analysis was conducted within a competing-risk model framework with the inference conducted on the basis of cause-specific hazard(17, 18). Cumulative incidence curves were constructed to visualize the influence of each of the top three hits with neuropathy incidence. The relationship between each SNP and neuropathy was adjusted for clinical covariates with putative relevance to neuropathy risk: diabetes (reported history of diabetes or current diabetes treatment vs. none), age (continuous), body mass index (BMI) ($\leq 30 \text{ kg/m}^2$ vs. $> 30 \text{ kg/m}^2$), and treatment arm (bevacizumab vs. placebo), regardless of whether the covariate was significantly associated with neuropathy in this cohort.

Cross-study clinical replication of the SNP with the greatest association with neuropathy was attempted in a cohort of Caucasian breast cancer patients treated with paclitaxel on the CALGB 40101 clinical trial(22). GWAS of the cumulative paclitaxel dose at occurrence of grade 2+ neuropathy has previously been published(9). Additionally, a SNP predictive of paclitaxel-induced neuropathy risk (rs10771973, *FGD4*) discovered and replicated in the CALGB 40101 GWAS was interrogated in the GWAS results from the docetaxel-treated Caucasian patients on CALGB 90401 to assess whether the SNPs that influence neuropathy risk are similar between the two taxanes.

Statistical analyses were performed by Alliance statisticians on a database locked on August 15, 2012. All analyses were conducted using the R [R]statistical environment version 3.1.1(23) or higher, using extension packages survival (version 2.37-7)(24), GenABEL (version 1.8-0)(25), cmprsk (version 2.2-7)(26) and interval (version 1.1-0.1)(27). To search for cumulative genetic effects, the genes that mapped to the top 1000 SNPs were included in a pathway enrichment analysis using the Ingenuity Pathway Analysis version 17199142 (Ingenuity® Systems, Inc, www.ingenuity.com) internal algorithm. Peripheral neuronal cell phenotypes (outgrowth, processes, branching, etc.) measured after treatment with increasing docetaxel and paclitaxel concentrations were compared between *VAC14* siRNA and non-targeted control siRNA using two-way ANOVA.

Mouse mechanical sensitivity thresholds were compared prior to docetaxel treatment between *VAC14*^{+/+} and *VAC14*^{+/-} mice using an interval-censored analysis with the minimum fiber size inducing a response as the phenotype. The genotype groups in the untreated mice were compared again on day 28. The same method was used to compare sensitivity thresholds of docetaxel treated and untreated mice following treatment on day 8. To assess whether *VAC14*^{+/-} mice were more sensitive to docetaxel-induced changes in mechanical stimuli, parametric survival regression was used to test for the effect of *VAC14* by treatment interaction on withdrawal threshold on day 8, after treatment was completed.

Results

Genome-wide Association Study

The CALGB/Alliance 90401 parent study enrolled 1,050 patients, of whom 863 provided consent and registered for the pharmacogenomics substudy CALGB 60604 (CALGB is now

a part of the Alliance for Clinical Trials in Oncology). The GWAS included 790 patients, of whom only the 616 self-reported, genetically defined Europeans receiving treatment were included in the analysis to minimize confounding from population substructure (Supplementary Figure 1). Demographic characteristics for the discovery cohort including baseline covariates potentially relevant to treatment-induced neuropathy risk are reported in Table 1. The median age was 69 years, body mass index (BMI) was 29 kg/m², and 97 patients (16%) had a history of diabetes. Randomization was nearly even to the bevacizumab (314 patients, 51%) and placebo (302, 49%) arms.

The overall incidence of grade 3+ sensory neuropathy was 8.1% (50/616). The 566 patients who did not experience neuropathy were classified as having either completed treatment without neuropathy (3.9%) or categorized based on their reason for discontinuation: death/progression (41.1%), non-CIPN treatment terminating adverse event (30.2%) or withdrawal/other (16.7%) (Supplementary Table 1). The risk of neuropathy was not significantly different in the bevacizumab and placebo arms (p=0.11), which were pooled for analysis.

GWAS analysis was performed on the cumulative docetaxel dose (mg/m²) at first report of treatment-induced grade 3+ sensory peripheral neuropathy (Figure 1). The ten most significant SNPs, ranked according to the unadjusted p-value for cause-specific association with neuropathy, are listed in Table 2 with rsID, gene annotation, and hazard ratios before and after adjustment (Top 1000 SNPs in Supplementary Table 2). One SNP surpassed Bonferonni-corrected significance (0.05/498,022=1.004×10⁻⁷). This is an intronic SNP in the *VAC14* gene (rs875858, minor allele frequency=0.056), which increased neuropathy risk (HR=3.60, 95%CI: 2.21-5.84, p=2.12×10⁻⁸, Figure 2A). The second and third most strongly associated SNPs were an intergenic SNP (rs11017056, MAF=0.223, HR=2.61, 95%CI: 1.77-3.84, p=3.84×10⁻⁷) and an intronic SNP in the *ATP8A2* gene (rs1326116, MAF=0.194, HR=2.77, 95%CI: 1.79-4.27, p=1.77×10⁻⁶), both of which increased neuropathy risk (Figures 2B and 2C).

The associations of all SNPs were then adjusted for treatment arm and several clinical covariates previously reported to be relevant to neuropathy risk including diabetes, age, and BMI. In this particular cohort, among the clinical covariates only increasing age (p=0.0003) was significantly associated with neuropathy, though increasing BMI showed a trend toward association (p=0.05). Covariate adjustment did not substantially affect the results, though the association of *VAC14* fell below the Bonferonni-corrected significance threshold after adjustment (p=5.88 ×10⁻⁷). The top 1000 SNPs mapped to 240 unique genes, which were included in the pathway analysis. The three most significantly enriched pathways in the GWAS are displayed in Supplementary Table 3. Notably, two of these enriched pathways are relevant to CIPN; Axonal Guidance (p=1.20×10⁻⁴) and Neuropathic Pain Signaling in Dorsal Horn Neurons (p=6.03×10⁻³).

Cross-GWAS Pharmacogenetic Replication

Due to the absence of a large cohort of docetaxel-treated patients with prostate cancer in which to perform replication, and our interest in assessing whether taxanes share genetic predictors of neuropathy risk, cross-study pharmacogenetic replication was attempted in a cohort of paclitaxel-treated Caucasian patients with breast cancer enrolled on CALGB

(Alliance 40101). The *VAC14* SNP (rs875858) discovered in our docetaxel-induced neuropathy GWAS was not associated with paclitaxel-induced neuropathy in the 40101 study ($p=0.70$)⁽⁹⁾. In addition, SNPs that were discovered in previously published GWAS of paclitaxel-induced neuropathy (*FGD4* (rs10771973) $p=0.39$, *EPHA4* (rs17348202) $p=0.76$, *EPHA5* (rs7349683) $p=0.15$, intergenic (rs3125923) $p=0.057$, *FCAMR* (rs1856746) $p=0.25$)^(9, 10, 12) or candidate SNP studies of docetaxel-induced neuropathy (*GSTP1* Ala114Val (rs1138272) $p=0.98$, *ABCB1* 1236C>T (rs1128503) $p=0.62$, *ABCB1* (rs4148738) $p=0.90$, *ABCB1* 3435C>T (rs1045642) $p=0.80$)⁽¹³⁻¹⁵⁾ were not associated with docetaxel-induced neuropathy occurrence in the CALGB 90401 patients. Finally, when comparing the top SNPs from taxane-induced neuropathy GWAS performed within CALGB 90401 (docetaxel) and 40101 (paclitaxel), there was no statistical evidence that the degree of overlap was greater than would be expected by random chance (data not shown).

Peripheral Neuronal Cell Sensitivity

To determine the effect of *VAC14* knockdown on neuronal sensitivity to docetaxel and paclitaxel, commercially available human iPSC-derived peripheral neurons (Peri4.U) were utilized. At the time of image analysis, which is 24 and 48 hours post drug treatment (48 and 72 h post transfection), expression of *VAC14* in neurons treated with 1 μ M siRNA had decreased to 54 and 44%, respectively, as compared to control cells treated with a non-targeting siRNA (Figure 3B). The *VAC14* siRNA knockdown at 24 hours post-docetaxel treatment did not affect relative total outgrowth (Figure 3C) in the peripheral neurons but resulted in significantly greater sensitivity to docetaxel as measured by relative number of processes ($p=0.0015$, Figure 3D) and relative number of branches ($p<0.0001$, Figure 3E). Similar effects were found in preliminary studies of *VAC14* knockdown in a human iPSC-derived cortical neuron, iCell[®] Neurons measured 48 hours after treatment (Cellular Dynamics International, Madison, WI) (data not shown). In contrast, *VAC14* siRNA knockdown significantly decreased peripheral neuronal cell sensitivity to paclitaxel as measured by these same cellular phenotypes (all $p<0.05$, Figure 3F-3H). Other morphological characteristics including mean process length, mean outgrowth intensity and relative straightness were also measured for docetaxel and paclitaxel (Supplementary Figure 2). We found a decreased sensitivity to docetaxel and paclitaxel when measuring mean process length and for mean outgrowth intensity for paclitaxel only. There was no difference in measurements of relative straightness (data not shown).

Mouse Mechanical Sensitivity

Prior to receiving docetaxel treatment, *VAC14*^{+/-} heterozygous mice were more sensitive than wild type *VAC14*^{+/+} mice to mechanical stimuli using von Frey filaments ($p=0.001$, Supplementary Figure 3). When stratified by gender this effect was significant in male mice ($p=0.002$) but not female mice ($p=0.16$), however, there was no statistically significant gender interaction ($p=0.10$). The difference in sensitivity to mechanical stimuli between genotype groups at baseline was attenuated over the course of the experiment in untreated mice, as there was no evidence of a difference between these groups from at the end of the experiment ($p=0.23$). As expected, 8 days of docetaxel treatment increased sensitivity to mechanical stimuli compared to untreated control mice, regardless of mouse genotype ($p<0.0001$, Figure 4). Comparing across genotype groups, there was no difference between

VAC14^{+/-} and *VAC14*^{+/+} mice in docetaxel-induced increase in threshold sensitivity to mechanical stimuli at day 8 (p=0.18) or day 14 (p=0.80) when the neuropathy was at its maximum.

Discussion

GWAS in a large cohort of mCRPC patients receiving docetaxel treatment identified an intronic SNP (rs875858) within *VAC14* that was associated with increase neuropathy risk (HR=3.60). *VAC14* has been directly linked to neurodegeneration in animal models(20) and linked to hereditary neuropathy conditions in humans(28). The lack of clinical replication in a cohort of paclitaxel-treated breast cancer patients is perhaps due to known differences between the two taxanes, including neuropathy profile(29). Distinct genetic modifiers of cellular sensitivity to paclitaxel and docetaxel have been previously reported in *in vitro* models(30) and the results of cellular sensitivity experiments demonstrating that *VAC14* knockdown enhances sensitivity of peripheral neurons to docetaxel but not paclitaxel for relative number of processes and relative branching, further support this hypothesis. Given the distinct genetic effects on neuronal sensitivity to docetaxel and paclitaxel it is conceivable that the polymorphisms that affect neuropathy risk also differ between the two taxanes. This would be consistent with the lack of cross-study replication, including the lack of association with docetaxel-induced neuropathy occurrence in this analysis for SNPs (*FGD4*, *EPHA4* and *EPHA5*) that have been previously discovered, and in some cases replicated, in paclitaxel-induced neuropathy GWAS (9, 10, 12, 31). Alternatively, heterogeneity between the patient cohorts, including differences in gender, age, and functional status, or differences in the phenotype (grade 2+ vs. grade 3+), may explain the lack of replication across the clinical GWAS. This GWAS was also not able to replicate previously reported associations between docetaxel-induced neuropathy and SNPs in *ABCB1*(15) or *GSTP1*(13, 14) reported in previous candidate SNP studies, similar to the failure of paclitaxel GWAS to replicate associations with candidate SNPs including *CYP2C8*3*(32).

The mechanistic importance of *VAC14* in docetaxel-induced neuropathy was tested in two pre-clinical models of CIPN. Knockdown of *VAC14* in the peripheral neuronal cell model (Peri.4U) increased sensitivity to docetaxel treatment as measured by decreased neurite number of processes and neurite branching. Interestingly, knockdown of *VAC14* seemed to decrease cellular sensitivity to paclitaxel treatment for these morphological characteristics suggesting that this gene may play an important role in the damage to processes and branching induced by docetaxel but not paclitaxel. A similar model of human neuronal cell sensitivity that utilizes induced pluripotent cortical neurons (iCell® Neurons) has previously been used for mechanistic validation of GWAS hits that increases risk of paclitaxel-induced neuropathy(19, 33) and vincristine-induced neuropathy(11). These human model systems have several advantages over the use of rodent cell lines such as rat pheochromocytoma cell lines, PC12 or NS1. One major limitation of rodent cell lines is the required treatment with nerve growth factor to initiate nerve outgrowth, which confounds the evaluation of drug treatment effects(34-36).

To our knowledge this is the first study to attempt mechanistic validation of a finding from a clinical GWAS in an animal model of CIPN. The Von Frey Filament Test is a well-established rodent model to test sensitivity to nociceptive stimuli, as a surrogate for peripheral neuropathy, that is commonly used for mechanistic investigation of neuropathy(21, 37, 38). Unlike the cell experiment, *VAC14* heterozygous mice were not more sensitive than wild-type littermates to the effect of docetaxel, but were more sensitive to mechanical stimuli prior to treatment. The enhanced sensitivity in the heterozygous mice at baseline was attenuated over time in the untreated cohort, in both male and female mice. It is possible that these mice acclimate to the discomfort of the mechanical stimuli or they may be experiencing numbness, another characteristic symptom of peripheral neuropathy in taxane-treated patients, though additional experiments would be required to test either hypothesis. It is unclear from these findings whether patients with *VAC14* polymorphisms have subclinical neuropathy at baseline that is expressed by treatment, as suggested by the mouse data, or whether they are similar at baseline and have greater neuropathy development from docetaxel treatment. Subjective testing that is sensitive for subclinical neuropathy, perhaps with electrophysiological or quantitative sensory assessment(39), in untreated patients with wild-type and polymorphic *VAC14* may help resolve this conundrum.

The *VAC14* protein provides a scaffold for the lipid kinase PIKfyve and its positive regulator, FIG4. These proteins reside in a complex that is responsible for the generation of the signaling lipids phosphatidylinositol (3,5)-bisphosphate (PI(3,5)P₂) and PI5P. These lipids are essential in multiple tissues(28). The inability of *VAC14*^{-/-} homozygous mice to survive beyond 1-2 days after birth necessitated the use of *VAC14*^{+/-} heterozygous mice(20). On autopsy *VAC14*^{-/-} homozygous mice exhibit massive neurodegeneration with cell bodies in the central and peripheral nervous system containing large vacuoles. This neurodegeneration and vacuolization are similar to that found in *FIG4* null mice, suggesting that variation in these genes is likely to phenocopy in humans as well as in mice(40). A rare mutation in the *FIG4* gene is known to cause a subtype of Charcot-Marie-Tooth disease, a hereditary neuropathy syndrome(41). Interestingly, the CALGB 40101 paclitaxel-induced neuropathy GWAS discovered and replicated an association for a SNP in *FGD4* (rs10771973), another gene known to cause Charcot-Marie-Tooth disease(42), though this variant was not associated with docetaxel-induced neuropathy in our CALGB 90401 cohort.

The SNP (rs875858) discovered in this GWAS lies within an intron of the *VAC14* gene. It is unclear what effect, if any, this individual SNP has on *VAC14* expression or activity in the peripheral neuron. In the GTEx database there is no association between rs875858 and *VAC14* gene expression in the tibial nerve tissue, though the power of this analysis is limited by the relatively small number of samples (n=256) and low minor allele frequency (MAF=0.06) of this SNP http://www.gtexportal.org/home/eqtls/bySnp?snpId=rs875858&tissueName=Nerve_Tibial. In the larger DGN cohort (n=922), rs875858 is highly associated with *IL34* (p=4e-7) expression in whole blood. *IL-34* is structurally similar to colony-stimulating factor 1 and both are ligands of the colony-stimulating factor 1 receptor(43), which has recently been identified as an efficacious target in mouse models of Charcot-Marie-Tooth disease(44), providing an alternative plausible biological mechanism for this association. Finally, it is conceivable that this SNP is not functionally relevant but

instead is a genetic marker of a different causative SNP; however, rs875858 lies in a region of low linkage disequilibrium.

The SNP (rs1326116) with the third strongest association with docetaxel-induced neuropathy is an intronic polymorphism in the *ATP8A2* gene, which encodes ATP8A2, a P4 ATPase. The P-type ATPases are a superfamily of proteins that translocate phospholipids between the two leaflets of cellular lipid bilayers(45). Several lines of evidence link ATP8A2 to neurological development and function; in vitro data that ATP8A2 overexpression enhances neurite outgrowth(46), animal data that *ATP8A2* mutations cause overt progressive neurodegeneration in Wobbler-lethal mice by disrupting axonal transport and polarity(47), and case reports of *ATP8A2* mutations causing severe neurological phenotypes in patients(48, 49).

The three pathways most highly enriched in the GWAS results include axonal guidance and neuropathic pain signaling, both of which have obvious relevance to taxane-induced peripheral neuropathy. Growth and maintenance of neuronal axons requires organization of microtubules into a cytoskeleton(50). Taxanes stabilize microtubules, potentially interfering with cytoskeletal organization leading to the overt neuropathic symptoms reported by patients. Enrichment in a similar pathway, axonal outgrowth, was reported in a secondary analysis of a paclitaxel-induced neuropathy GWAS, suggesting that these two studies are identifying similar genetic signatures(51), despite the lack of overlap of individual SNPs in the top 1000 hits. Neuropathic pain is one of the characteristic clinical manifestations of taxane-induced neuropathy, in addition to sensory and motor components(2). Neuropathic pain signaling in the dorsal horn neurons may contribute to taxane-induced peripheral neuropathy via a higher propensity for development of central sensitization(52).

The primary limitation of this study is the lack of availability of an independent clinical trial cohort of docetaxel-treated patients for pharmacogenetic replication. Another limitation is the reliance on the protocol-specified collection of severe peripheral neuropathy (grade 3 or higher), rather than systematic collection across all toxicity grades. It is possible that some patients experienced grade 2 neuropathy without progressing to grade 3, and thus were not included as neuropathy events in this analysis. Furthermore, ordinal analysis across all neuropathy grades (0-4) would have brought greater statistical power and may have identified a distinct set of SNPs that predict neuropathy severity, similar to the paclitaxel-induced neuropathy GWAS findings in CALGB (Alliance) 40101(9). The lack of grade 1 or 2 neuropathy data collection may explain the absence of events in the patients homozygous for the rs875858 SNP. However, two of these patients discontinued docetaxel treatment quite early, after 6 (442 mg/m²) and 8 (561 mg/m²) cycles for progression and grade 3 nausea/vomiting, respectively. The third patient was discontinued after 26 cycles (1,746 mg/m²) for a constellation of grade 3 toxicities including cough, dyspnea, fatigue, febrile neutropenia, and pneumonitis, without any documentation of peripheral neuropathy during treatment. The collection of DNA and comprehensive baseline and outcomes data, including accurate assessment of timing and severity of peripheral neuropathy, in other large clinical cohorts will allow for independent replication of our findings.

In conclusion, GWAS of a prospectively enrolled patient cohort identified a SNP in *VAC14* that predicts sensitivity to docetaxel-induced peripheral neuropathy. *VAC14* heterozygous mice were subsequently shown to be more sensitive to nociceptive stimuli at baseline, and peripheral neuronal cell (Peri.4U) sensitivity to docetaxel is enhanced after *VAC14* knockdown, providing mechanistic validation of the relevance of *VAC14* to docetaxel-induced neuropathy. However, the relatively low hazard ratio of this SNP suggests that no single variant or gene is responsible for dictating neuropathy sensitivity or will be clinically useful for personalizing docetaxel treatment. This is consistent with the multifactorial nature of neuropathy that depends on both drug exposure and a variety of patient factors, including age and BMI, which were associated with neuropathy occurrence in this cohort. Replication of the influence of our top candidate SNPs on neuropathy risk in a similar clinical trial cohort of docetaxel-treated mCRPC patients is of great interest to validate the role of these genes and provide support for further investigation into this mechanism for chemotherapy-induced neuropathy prevention or treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of Translational Relevance

The taxanes are a class of chemotherapeutic agents that are commonly associated with treatment-limiting peripheral sensory neuropathy. Genetic predictors of paclitaxel-induced neuropathy have been discovered but little is known about whether similar genetic factors are associated with risk of docetaxel-induced neuropathy. Discovery of genetic predictors of docetaxel-induced neuropathy could inform personalized treatment decisions to maximize efficacy and avoid unnecessary toxicity. We conducted a genome-wide association study in a large cohort of Caucasian men with hormone refractory prostate cancer treated with docetaxel. One single nucleotide polymorphism in *VAC14* that was significantly associated with docetaxel-induced neuropathy was then mechanistically validated in cellular and animal models of neurotoxicity. These findings suggest that distinct genetic variants influence risk of peripheral neuropathy from paclitaxel and docetaxel. The genetic variant identified in this study could be useful for understanding the mechanism of docetaxel-induced neuropathy and may be informative for avoiding docetaxel treatment in patients at elevated neuropathy risk.

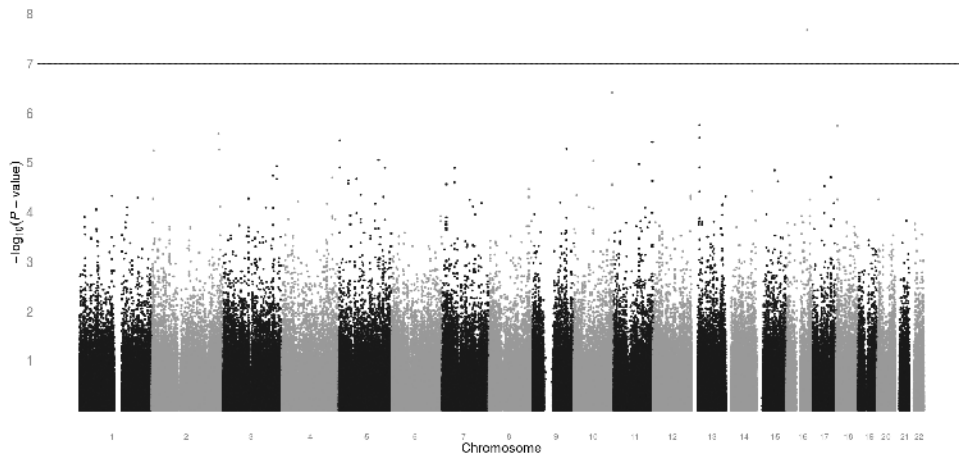
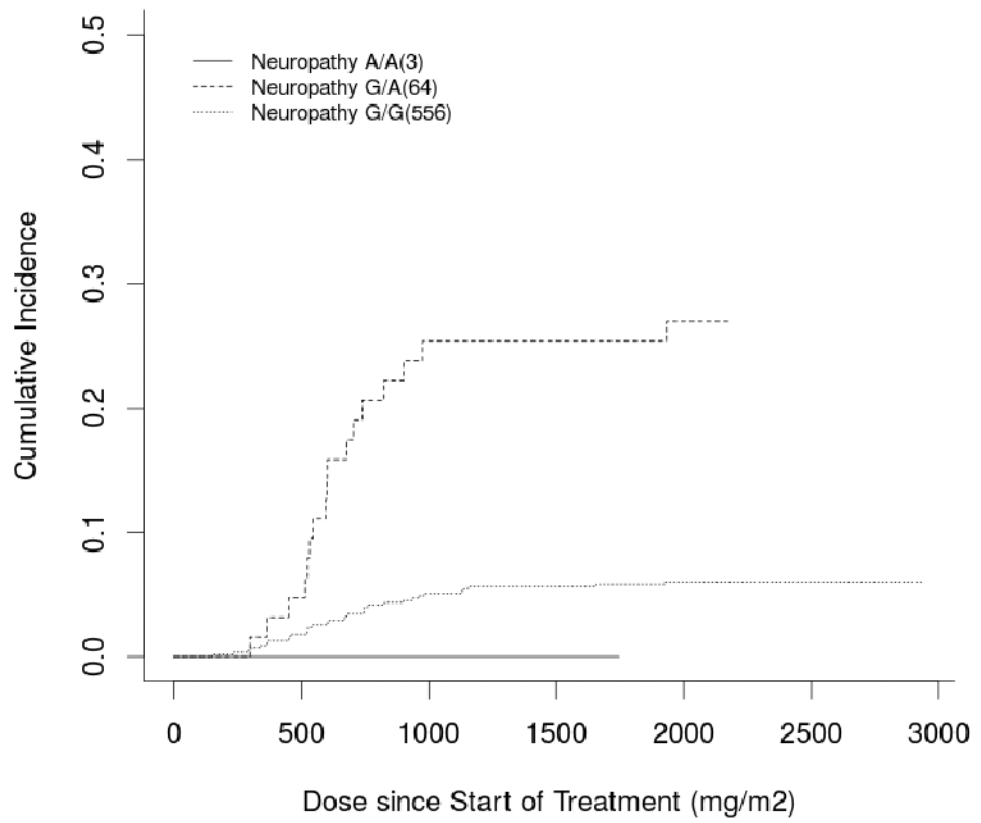


Figure 1. Genome-wide association results

Manhattan plot displaying the unadjusted p-value for the association of cumulative docetaxel dose at grade 3+ sensory neuropathy for all SNPs that passed quality control (n=498,022). One SNP (rs875858) surpassed the Bonferonni-corrected significance threshold of 1.0×10^{-7} .

rs875858



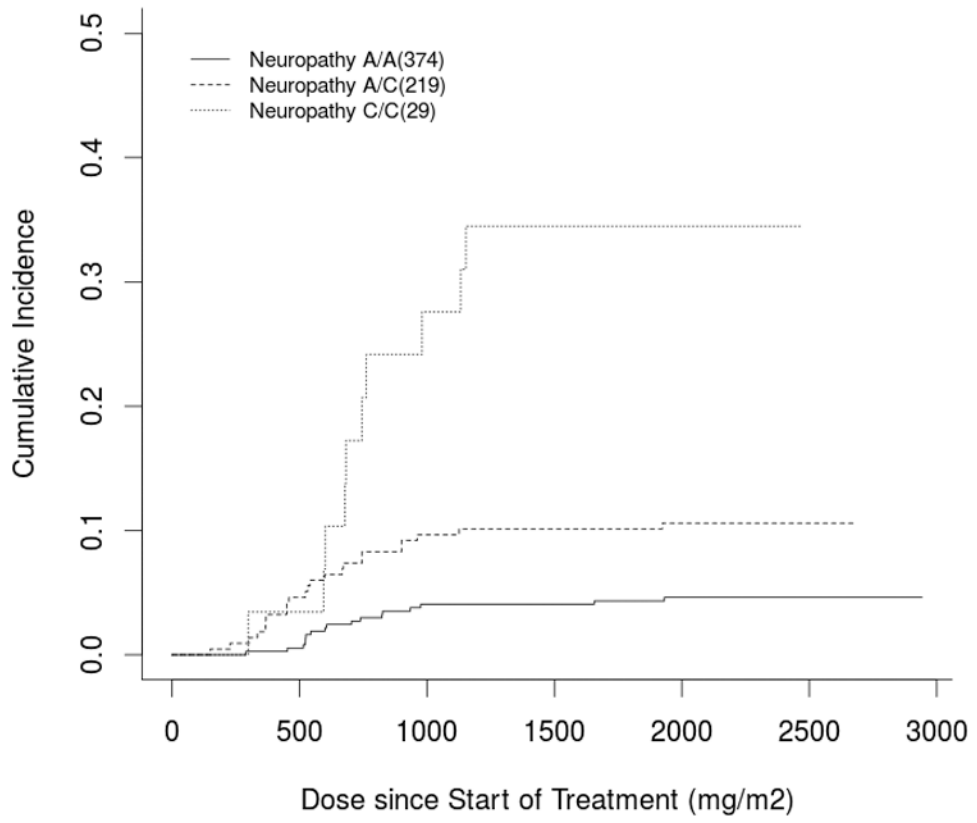
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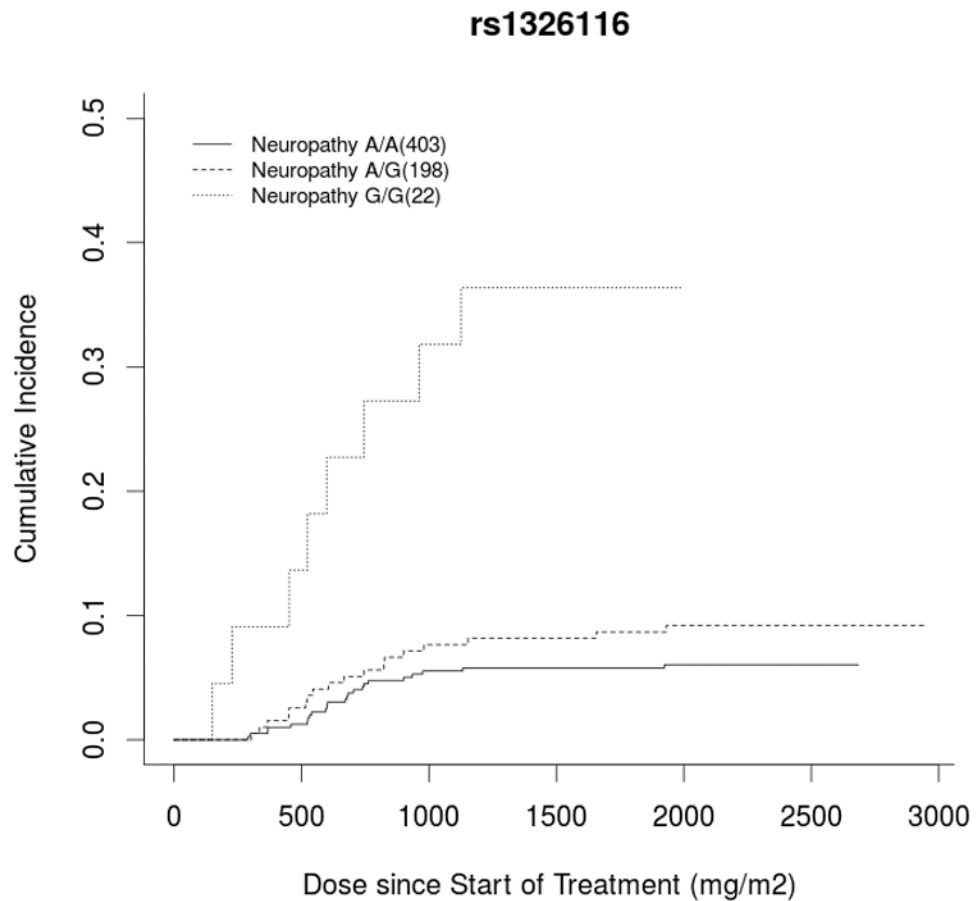


Figure 2. Incidence of Neuropathy by Genotype for Highly Associated SNPs

Cumulative incidence curve of docetaxel dose at grade 3+ neuropathy occurrence stratified by genotype for the top three hits: A) a *VAC14* intronic SNP (rs875858), B) an intergenic SNP (rs11017056), and C) an *ATP8A2* intronic SNP (rs1326116). In each case, carrying the variant allele increased neuropathy risk compared to wild-type patients, though there were too few patients who were homozygous for the rare *VAC14* variant ($n=3$) to draw meaningful conclusions in this group.

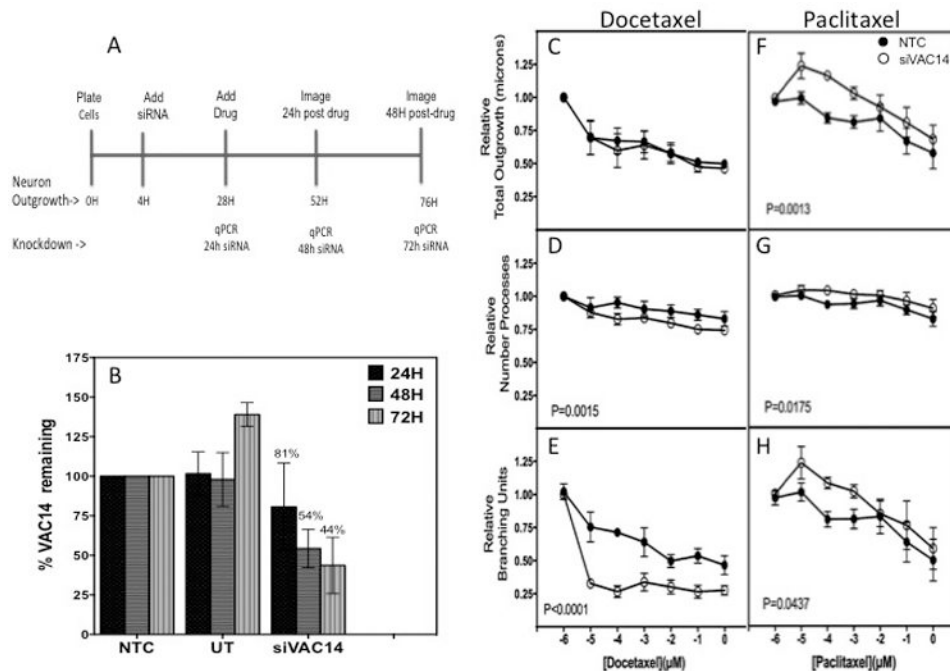


Figure 3. VAC14 Knockdown increases neuronal cell sensitivity to docetaxel but decreases sensitivity to paclitaxel

A) Peripheral neuron (Peri.4U) experimental scheme illustrating timing of treatment and imaging. B) Relative VAC14 expression at 24, 48 and 72 hours post-transfection with siVAC14 compared to non-targeting control (NTC) produced a steady decline of VAC14 expression over time. The neurite outgrowth changes after 24 hours docetaxel (48 hours knockdown) showed no significance as measured by C) total outgrowth but did result in greater sensitivity to docetaxel in D) relative number of processes and E) relative number of branches. Alternatively, VAC14 knockdown decreased peripheral neuronal cell sensitivity to paclitaxel, 3F-3H. Open circle represent data from VAC14 siRNA and closed circle from NTC siRNA. Neurite parameters measured with MetaXPress® software were averaged from 3 independent experiments. Error bars represent the standard error of the mean from three independent transfection experiments. NTC = non-targeting control, UT= untransfected control.

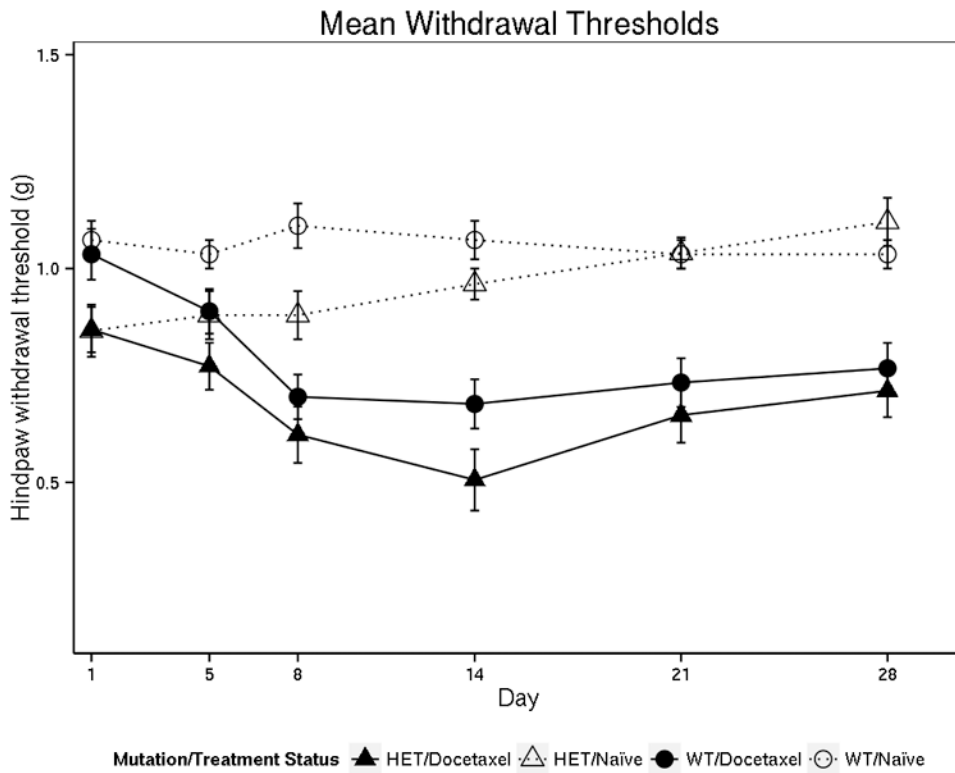


Figure 4. Docetaxel Treatment Increases Nociceptive Sensitivity in *VAC14* Heterozygous and Wild-type Mice

The withdrawal threshold is defined as the lightest fiber weight that caused each mouse to withdraw their hind paw at least three times out of five. Each mouse has a withdrawal threshold at each time-point in the study. The mean withdrawal threshold is the average of 12-14 mice (6-7 for each gender) for each treatment condition (*VAC14* genotype and docetaxel treatment). Docetaxel-treated mice of either genotype had greater hindpaw withdrawal sensitivity following the end of treatment at day 8 compared to the untreated control mice ($p < 0.0001$). There was no significant difference in the docetaxel-induced increase in withdrawal sensitivity comparing *VAC14* heterozygous and wild-type mice ($p = 0.18$).

Table 1

Demographic Data for Patients Included in GWAS (n=616).

Age	Median (years)	68.9
	Range (years)	41.7-93.5
Body mass index	Median (kg/m ²)	29.0
	Range (kg/m ²)	16.1-53.3
Diabetes	Yes	97 (15.8%)
	No	519 (84.3%)
Treatment arm	Bevacizumab	314 (51.0%)
	Placebo	302 (49.0%)
Cumulative docetaxel dose	Median (mg/m ²)	626
	Range (mg/m ²)	72-2,942
ECOG performance status	0	340 (55.2%)
	1	258 (41.9%)
	2	18 (2.9%)
Disease type	Measurable disease	310 (50.3%)

Table 2

Ten SNPs Most Associated with Neuropathy in GWAS

rsID	Chr	Position	Gene	MAF	P-value	HR	95% CI	Adjusted*	
								P-value	HR
rs875858	16	69332956	VAC14	0.056	2.12E-08	3.60	2.21-5.84	5.88E-07	3.85
rs11017056	10	131720630	Intergenic	0.223	3.84E-07	2.61	1.77-3.84	6.36E-07	2.58
rs1326116	13	25273046	ATP8A2	0.194	1.77E-06	2.77	1.79-4.27	4.57E-06	2.99
rs8089250	18	4762807	Intergenic	0.175	1.83E-06	2.72	1.78-4.17	2.51E-05	2.49
rs6436559	2	226061042	KIAA1486	0.063	2.62E-06	3.34	1.95-5.71	3.26E-05	3.19
rs11840337	13	25287298	ATP8A2	0.191	3.16E-06	2.71	1.75-4.18	9.26E-06	2.90
rs1566691	5	3780179	Intergenic	0.057	3.63E-06	3.64	2.03-6.52	4.45E-05	3.41
rs1027796	11	132262880	OPCML	0.305	3.90E-06	2.38	1.62-3.48	2.41E-05	2.32
rs2213920	9	117311405	Intergenic	0.128	5.26E-06	2.63	1.70-4.06	2.11E-05	2.66
rs4438470	2	228722963	SPHKAP	0.137	5.47E-06	2.71	1.73-4.25	7.79E-05	2.47

95% CI: 95% Confidence interval around hazard ratio estimate

Chr: Chromosome

HR: Hazard ratio

MAF: minor allele frequency in the 90401 Caucasian cohort

* Adjusted for patient BMI, age, history of diabetes, and treatment arm.